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The TRA-1A Sex Determination Protein of *C. elegans* Regulates Sexually Dimorphic Cell Deaths by Repressing the *egl-1* Cell Death Activator Gene

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Summary

The hermaphrodite-specific neurons (HSNs) of the nematode *Caenorhabditis elegans* are generated embryonically in both hermaphrodites and males but undergo programmed cell death in males. The gene *egl-1* encodes a BH3-containing cell death activator that is required for programmed cell death in *C. elegans*. Gain-of-function (gf) mutations in *egl-1* cause the inappropriate programmed cell death of the HSNs in hermaphrodites. These mutations lie 5.6 kb downstream of the *egl-1* transcription unit and disrupt the binding of the TRA-1A zinc finger protein, the terminal global regulator of somatic sexual fate. This disruption results in the activation of the *egl-1* gene in the HSNs not only in males but also in hermaphrodites. Our findings suggest that in hermaphrodites TRA-1A represses *egl-1* transcription in the HSNs to prevent these neurons from undergoing programmed cell death.

Introduction

Programmed cell death is a fundamental feature of animal development, and the mechanisms of programmed cell death have been conserved through evolution (reviewed by Ellis et al., 1991; Jacobson et al., 1997). Programmed cell death is necessary for normal neurogenesis in both vertebrates and invertebrates (reviewed by Oppenheimer, 1991; Pettmann and Henderson, 1998) and can result in sexual dimorphism within the nervous system (Truman, 1984; Oppenheimer, 1991). For example, a greater extent of programmed cell death in the development of the female nervous system contributes to the formation of sexually dimorphic structures in the song control region of the zebrafinch brain (Konishi and Akutagawa, 1985; Kirn and DeVoogd, 1989) and in the preoptic area and the bulbocavernosus in mammals (Nordeen et al., 1985; Breedlove, 1986; Davis et al., 1996). In the moth *Manduca sexta* programmed cell death is required for the formation of the sexually dimorphic pattern of imaginal midline neurons (Thorn and Truman, 1994).

Sexual dimorphism also exists in the nervous system of the nematode *Caenorhabditis elegans*. The nervous system of the *C. elegans* hermaphrodite consists of 302 neurons, eight of which are found only in hermaphrodites. *C. elegans* males, on the other hand, have a total of 381 neurons, 87 of which are found only in males (reviewed by Hodgkin, 1988). Most of the sexually dimorphic neurons are generated by differential numbers of cell divisions or by differential cell fates. However, a few sex-specific neurons, such as the hermaphrodite-specific neurons (HSNs), are a consequence of sexually dimorphic programmed cell death. The HSNs, a pair of serotonergic motor neurons required for egg laying by hermaphrodites, are generated embryonically in both hermaphrodites and males but undergo programmed cell death in males shortly after they are formed (Sulston and Horvitz, 1977; Sulston et al., 1983).

Genetic analyses in *C. elegans* have lead to the identification of four genes, *ced-3*, *ced-4*, *ced-9*, and *egl-1* (*ced*, *c*ell-*d*eath defective; *egl*, *eg*g-*l*aying defective), that can mutate to block programmed cell death and thus appear to be components of a central programmed cell death pathway (reviewed by Metzstein et al., 1998). The gene *ced-3* encodes a member of the caspase family of cysteine proteases (reviewed by Nicholson and Thornberry, 1997), *ced-4* encodes a protein with similarities to the mammalian cell death activator Apaf1 (reviewed by Vaux, 1997), *ced-9* encodes a Bcl2-like cell death inhibitor (reviewed by Adams and Cory, 1998), and *egl-1* encodes a BH3 domain–containing protein similar in structure to the mammalian "BH3 domain–only" cell death activators, which form a subgroup within the family of Bcl2-like molecules (reviewed by Kelekar and Thompson, 1997; Adams and Cory, 1998). Genetically *ced-3*, *ced-4*, *ced-9*, and *egl-1* appear to act in a simple pathway in which *egl-1* negatively regulates *ced-9*, *ced-9* negatively regulates *ced-4*, and *ced-4* positively regulates *ced-3* (reviewed by Metzstein et al., 1998). It has been proposed that the EGL-1 protein activates programmed cell death by binding to and thereby negatively regulating the cell death inhibitor protein CED-9, releasing CED-4 from CED-9 and resulting in the CED-4-dependent activation of the CED-3 caspase (Conradt and Horvitz, 1998; del Peso et al., 1998).

Sexual fate in *C. elegans* is determined by the ratio of the number of X chromosomes to the number of sets of autosomes (X:A ratio) (reviewed by Meyer, 1997). This primary signal is transmitted through a cascade of interacting genes and determines the level of activity of the terminal, global control gene of somatic sex determination, *tra-1* (*tra*, transformer) (reviewed by Hodgkin, 1988; Meyer, 1997). *tra-1* activity is regulated posttranslationally (de Bono et al., 1995), and *tra-1* acts cell autonomously to promote female development of the soma (Hodgkin, 1987; Hunter and Wood, 1990). The presence of two X chromosomes (XX) results in high levels of *tra-1* activity and, as a consequence, in the formation of an animal with a female soma, specifically, a hermaphrodite. (*C. elegans* hermaphrodites are basically female animals that produce sperm during the L4 stage.) The presence of only one X chromosome (X0) results in low levels of *tra-1* activity and, therefore, in the development of an animal with a male soma, that is, a male (Hodgkin, 1987).

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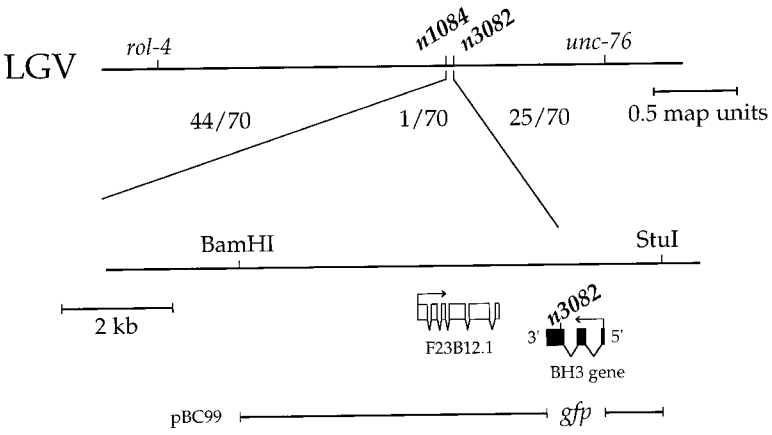


Figure 1. *n1084* and *n3082* Map within 0.04 Map Units of Each Other

(Top) Genetic map of a portion of the right arm of chromosome V and (Middle) corresponding physical map showing the predicted transcription units of the BH3 gene and an adjacent gene, F23B12.1. Genes and mutations used for the four factor–mapping experiment are indicated. Numbers below the genetic map represent the fraction of the recombination events that occurred between particular loci found in the 70 non-Rol Unc recombinants identified between *rol-4* and *unc-76*. (Bottom) 5′ and 3′ regulatory regions of the BH3 gene that were used to construct the P*egl-1::gfp* reporter construct pBC99 and the similar p*egl-1* (gf)::*gfp* construct pBC104 are indicated.

The *tra-1* gene encodes a DNA-binding protein, TRA-1A, with five zinc fingers most similar to the zinc fingers of the gene products of the segment polarity gene *cubitus interruptus* (ci) and the pair-rule gene *odd-paired* of *Drosophila* and the vertebrate Gli genes, which together form a subfamily within the family of Krüppel-like zinc finger DNA-binding proteins and transcription factors (Zarkower and Hodgkin, 1992; Benedyk et al., 1994). TRA-1A protein binds DNA in vitro in a sequence-specific manner, and it has been proposed that TRA-1A controls sexual fate by transcriptionally activating female-specific and/or by transcriptionally repressing male-specific genes required for sexual differentiation (Zarkower and Hodgkin, 1993). A number of genes have been identified that when mutated result in sex-specific defects (e.g., the *mab* genes [*mab*, *m*ale *ab*normal]; Hodgkin, 1983), and that therefore might be targets of TRA-1A. However, none of these genes has been shown to be a direct target of TRA-1A.

In this paper, we present data indicating that in the sexually dimorphic HSNs, the cell death activator gene *egl-1* is under the direct control of the *C. elegans* sex determination pathway. *egl-1*(gf) mutations cause the inappropriate deaths of the HSNs in hermaphrodites (Trent et al., 1983; Ellis and Horvitz, 1986). These *egl-1*(gf) mutations disrupt a TRA-1A-binding site 5.6 kb downstream of the *egl-1* transcription unit and result in the inappropriate activation of the *egl-1* gene in the HSNs in hermaphrodites.

Results

Dominant gain-of-function (gf) mutations in the *egl-1* gene cause the HSNs to inappropriately undergo programmed cell death in hermaphrodites (Trent et al., 1983; Ellis and Horvitz, 1986). Seven such *egl-1* mutations have been identified in screens for egg laying–defective mutants (Trent et al., 1983; Desai and Horvitz, 1989; J. Yuan et al., unpublished observations). In a screen for suppressors of the egg laying defect caused by the *egl-1*(gf) mutation *n1084*, we identified the mutation *n3082* (Conradt and Horvitz, 1998). This mutation suppresses the *egl-1*(*n1084*)-induced programmed cell deaths of the HSNs, because it results in the loss of function of a gene encoding a BH3-containing cell death activator required for programmed cell death (Conradt and Horvitz, 1998). The *n3082* mutation is linked to the *egl-1*(*n1084*gf) mutation, which raised the possibility that the *n3082* and *n1084* mutations affect the same gene. However, we did not find any mutations in the coding region or in the 5′ and 3′ untranslated regions of the BH3 gene in animals carrying any of the seven *egl-1*(gf) mutations. We show below that the *egl-1*(gf) mutations reside in regulatory regions of the BH3-containing cell death activator gene defined by *n3082*.

n1084 Maps about 0.04 Map Units Left of *n3082*

The *egl-1*(gf) mutation *n1084* and the *n3082* mutation both map to linkage group V between the genetic markers *rol-4* (*rol*, *rol*ler) and *unc-76* (*unc*, *un*coordinated), which are 2.7 map units apart (Conradt and Horvitz, 1998). To determine how tightly the two mutations are linked, we attempted to separate them using the *egl-1*(*n1084*gf) *n3082* chromosome, which we identified in our *egl-1*(*n1084*gf) suppressor screen, in a four factor–mapping experiment. Starting with a strain of genotype *rol-4 unc-76*/*egl-1*(*n1084*gf) *n3082*, we screened for Rol non-Unc and Unc non-Rol progeny. Among 70 identified Unc non-Rol progeny, one was of the phenotype Egl Unc non-Rol. The genotype of the recombinant chromosome of this animal was *egl-1*(*n1084*gf) *unc-76*, indicating that the recombination event had occurred between *egl-1*(*n1084*gf) and *n3082*. Among 137 identified Rol non-Unc progeny, none had an Egl phenotype. These findings indicate that the *egl-1*(*n1084*gf) mutation maps to the left of *n3082* on the genomic map or downstream of *n3082* on the physical map (Figure 1). The frequency of recombination between *egl-1*(*n1084*gf) and *n3082* detected among the Unc non-Rol progeny (1/70) suggests that *egl-1*(*n1084*gf) and *n3082* are approximately 0.04 map units apart and therefore tightly linked.

Identification of *egl-1*(gf) Mutations

These map data suggest that the *egl-1*(gf) mutations might affect downstream regulatory regions of the BH3 gene defined by *n3082*. The *egl-1*(gf) mutations cause a dominant phenotype. We therefore reasoned that DNA fragments that span the transcription unit of the BH3 gene and in addition carry an *egl-1*(gf) mutation downstream of this transcription unit might be capable of phenocopying the *egl-1*(gf) phenotype when introduced into hermaphrodites. To test this hypothesis, we used

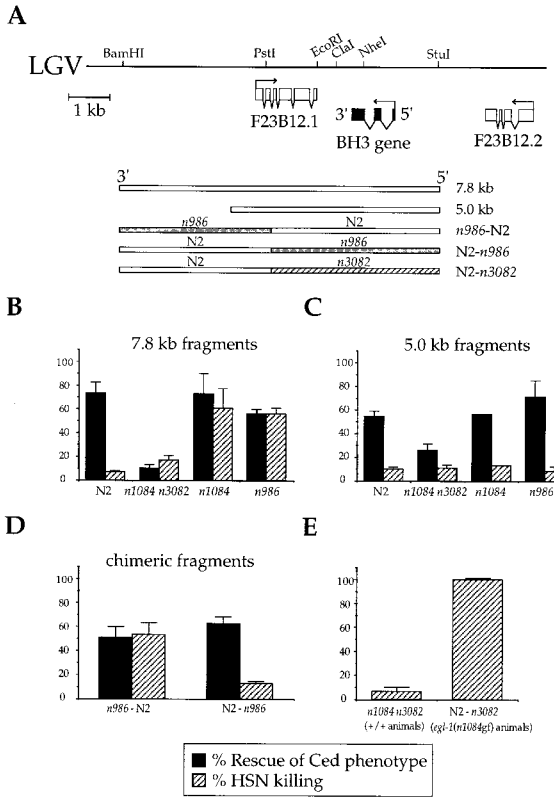


Figure 2. DNA Fragments Carrying *egl-1*(gf) Mutations Can Induce HSN Killing

(A) DNA fragments that were amplified by PCR are shown aligned with the physical map of the *egl-1* region.

(B) Fragments (7.8 kb) amplified from *egl-1*(gf) animals induced HSN killing. The fragments indicated were amplified by PCR and introduced by germline transformation into hermaphrodites of genotype *ced-1*(*e1735*); *egl-1*(*n1084 n3082*) *unc-76*(*e911*), as described in Experimental Procedures. Rescue of the Ced phenotype was analyzed by counting the number of cell corpses in the heads of transgenic larvae at the first larval stage using Nomarski optics, as described (Conradt and Horvitz, 1998). HSN survival was scored in transgenic animals at the first larval stage using Nomarski optics, as described (Conradt and Horvitz, 1998). Percentages reflect the abilities of various 7.8 kb fragments to rescue the Ced phenotype of the injected animals (black columns) and to induce HSN killing (hatched columns). The data are averages ± standard deviations of results obtained from six (N2), eight (*n1084 n3082*), four (*n1084*), and 19 (*n986*) independent transgenic lines.

(C) Fragments (5.0 kb) amplified from *egl-1*(gf) animals failed to induce HSN killing. The fragments indicated were generated by PCR, introduced into *ced-1*(*e1735*); *egl-1*(*n1084 n3082*) *unc-76*(*e911*) animals, and analyzed for their abilities to cause killing as described in (B). The data are averages ± standard deviations of results obtained from six (N2), four (*n1084 n3082*), one (*n1084*), and four (*n986*) independent transgenic lines.

(D) The 3′ 3.6 kb of fragments amplified from *egl-1*(gf) animals was sufficient to induce HSN killing. The 7.8 kb chimeric fragments indicated were made as described in Experimental Procedures. Transgenic lines were generated using *ced-1*(*e1735*); *egl-1*(*n1084 n3082*) *unc-76*(*e911*) animals and analyzed as described in (B). The data are averages ± standard deviations of results obtained from four (*n986*-N2) and seven (N2-*n986*) independent transgenic lines.

(E) The *egl-1*(gf) mutations were required in *cis* to a functional transcription unit of the BH3 gene to induce HSN killing. The fragments indicated were generated as described in Experimental Procedures, and data were collected as described in (B). The 7.8 kb *n1084 n3082* fragment was injected into animals of the genotype *unc-76*(*e911*) to generate transgenic lines. (In this case *n1084* was in *trans* to the wild-type transcription unit of the BH3 gene.) Data shown are averages ± standard deviations of results obtained from 12 independent transgenic lines. The 7.8 kb chimeric fragment N2-*n3082* was injected into animals of the genotype *egl-1*(*n1084*) *unc-76*(*e911*). (In this case *n3082* is in *trans* to *n1084*.) Data represent averages ± standard deviations of results obtained from 11 independent transgenic lines.

the polymerase chain reaction (PCR) to amplify DNA fragments from animals carrying *egl-1*(gf) mutations and analyzed these fragments for their abilities to induce the killing of the HSNs in hermaphrodites. We chose to amplify a 7.8 kb fragment that includes the transcription unit of the BH3 gene and 5.8 kb of its downstream region (Figure 2A). A wild-type genomic fragment of this length almost completely rescued the cell death defect caused by the *n3082* mutation and hence appears to include most regulatory regions of the BH3 gene (Conradt and Horvitz, 1998). We amplified the 7.8 kb fragment from the wild-type (N2) *C. elegans*, from *egl-1*(*n1084*gf) *n3082* animals, and from two strains carrying different *egl-1*(gf) mutations, *n1084* or *n986*. Using germline transformation, we introduced these fragments into *egl-1*(*n1084*gf) *n3082* animals, which are cell death defective (Ced) and have HSNs. Thus, we could score the fragments not only for their abilities to induce HSN killing but also for their abilities to rescue the Ced phenotype of *egl-1*(*n1084*gf) *n3082* animals as a control. The fragment amplified from wild-type animals rescued the Ced phenotype of the *egl-1*(*n1084*gf) *n3082* animals but did not induce the HSNs to undergo programmed cell death in hermaphrodites (Figure 2B). The fragment derived from *egl-1*(*n1084*gf) *n3082* animals, as expected, failed to rescue the Ced phenotype and did not induce HSN killing. By contrast, the fragments amplified from *egl-1*(*n1084*gf) or *egl-1*(*n986*gf) animals rescued the Ced phenotype and induced HSN killing in hermaphrodites (Figure 2B). The observed HSN killing was suppressed by a *ced-9*(gf) mutation, which blocks programmed cell death, confirming that the HSNs died by programmed cell death (data not shown). These results demonstrate that the 7.8 kb fragments amplified from *egl-1*(gf) mutants were able to phenocopy the *egl-1*(gf) dominant phenotype and therefore included the dominant *egl-1*(gf) mutations.

We then tested a 5.0 kb fragment that spans only the proximal 3.0 kb of the region downstream of the transcription unit of the BH3 gene (Figure 2A). Amplified from *egl-1*(gf) animals, these fragments still rescued the Ced phenotype of *egl-1*(*n1084*gf) *n3082* animals but were unable to induce HSN killing in hermaphrodites (Figure 2C). Furthermore, a 7.8 kb fragment composed of the 3′ 3.6 kb amplified from *egl-1*(*n986*gf) animals and the 5′ 4.2 kb from the wild-type strain (Figure 2A) was capable of inducing HSN killing (Figure 2D), while a fragment containing the wild-type 3′ 3.6 kb and the *egl-1*(*n986*gf) 5′ 4.2 kb (Figure 2A) failed to do so (Figure 2D). These results suggest that the *egl-1*(gf) mutations are likely to be located in the 3′ region of the 7.8 kb fragment.

We determined the nucleotide sequence of the 7.8 kb fragment for each of the seven *egl-1*(gf) mutants and

Table 1. The *egl-1*(gf) Mutations Are Single-Base Changes in a Putative Conserved TRA-1-Binding Site

		Core	
TRA-1-binding site		TTTCNNNNTGGGTGGTC	
C. briggsae egl-1 locus	+3801 bp	TTACGCACCGGGTGGTC	+3793 bp
C. elegans egl-1 locus	+5645 bp	CTCCTAACCGGGTGGTC	+5629 bp
n487, n1084, n1796		CTCCTAACCGGGTG**A**TC	
n986, n987, n2164		CTCCTAACCGG**A**TGGTC	
n2248		CTCCTAACCG**A**GTGGTC	

The consensus sequence of the TRA-1-binding site was determined by in vitro selection by Zarkower and Hodgkin (1993). The sequences of the putative TRA-1-binding site in the *egl-1* locus of *C. elegans* and *C. briggsae* were determined by the *C. elegans* Sequencing Consortium. The sequences of the TRA-1-binding sites of the *egl-1* locus of *egl-1*(gf) mutants were determined as described in Experimental Procedures.

found DNA lesions in all seven mutants. As expected, these lesions are at the 3′ end of the 7.8 kb fragment, clustered within five bp of each other 5631 to 5635 bp downstream of the stop codon of the BH3 gene. We found a G-to-A transition at bp +5631 in animals carrying the *egl-1*(gf) mutations *n487*, *n1084*, or *n1796*, a G-to-A transition at bp +5634 in animals carrying the *egl-1*(gf) mutations *n986*, *n987*, or *n2164*, and a G-to-A transition at bp +5635 in animals carrying the *egl-1*(gf) mutation *n2248* (Table 1).

The *egl-1*(gf) Mutations and *n3082* Are Allelic

The 7.8 kb fragment amplified from *egl-1*(*n1084*gf) animals but not from *egl-1*(*n1084*gf) *n3082* animals induced the HSNs to undergo programmed cell death (Figure 2B). This result indicates that an intact coding region of the BH3 gene is required for the ability of the *egl-1*(gf) mutations to induce HSN killing. Furthermore, the fact that the 7.8 kb fragment amplified from wild-type animals did not cause HSN deaths when injected into *egl-1*(*n1084*gf) *n3082* animals (Figure 2B), or likewise, the fact that a 7.8 kb fragment amplified from *egl-1*(*n1084*gf) *n3082* animals failed to cause HSN killing in wild-type (+/+) animals (Figure 2E) demonstrates that the *egl-1*(gf) mutations and an intact coding region of the BH3 gene must be in *cis* to cause HSN killing. Furthermore, the *n3082* mutation failed to suppress the *egl-1*(gf) mutation *n1084* when in *trans*: a chimeric 7.8 kb fragment carrying only the *n3082* mutation (Figure 2A) was unable to suppress HSN killing when introduced into *egl-1*(*n1084*gf) animals (Figure 2E). These observations indicate that the *n3082* mutation and the *egl-1*(gf) mutation *n1084* affect the same transcription unit, the transcription unit encoding the BH3 containing cell death activator, and therefore are allelic. The *egl-1* gene and the BH3 gene, hence, are the same and hereafter will be referred to as *egl-1*. *n3082* consequently represents a loss-of-function (lf) mutation in the *egl-1* gene, *egl-1*(*n1084 n3082*lf).

The *egl-1*(gf) Mutations Disrupt a Conserved Putative TRA-1A-Binding Site

The *egl-1*(gf) mutations are located within a stretch of nucleotides the sequence of which is highly similar to the core sequence of a DNA-binding site for the terminal, global regulator of somatic sex in *C. elegans*, TRA-1A (Zarkower and Hodgkin, 1993) (Table 1). This putative TRA-1A-binding site is located 5629 to 5637 bp downstream of the *egl-1* stop codon. The three different G-to-A transitions found in the seven *egl-1*(gf) mutants are within this core sequence and are predicted to severely affect the ability of TRA-1A to bind (Zarkower and Hodgkin, 1993). To determine whether this putative TRA-1A-binding site has been conserved through evolution, we cloned the *egl-1* locus from *C. briggsae*, a related *Caenorhabditis* species (our unpublished observations). We found an identical nine bp core sequence (and additional conserved areas) 3794 to 3802 bp downstream of the predicted stop codon of the *C. briggsae egl-1* gene (Table 1). This result suggests that this putative TRA-1A-binding site is important for the transcriptional regulation of a target gene. Our finding that this site is required in *cis* for the proper regulation of *egl-1* in the HSNs suggests that the *egl-1* gene represents a target of this conserved putative TRA-1A-binding site.

egl-1(gf) Mutations Cause the Inappropriate Activation of *egl-1* in the HSNs in Hermaphrodites

To determine the expression of the *egl-1* gene in the HSNs and the effect that the *egl-1*(gf) mutations in the conserved putative TRA-1A-binding site have on this expression, we made a green fluorescent protein (GFP) reporter construct in which the expression of the *gfp* gene is under the control of the 5′ (1.0 kb upstream of the 5′ UTR of *egl-1*) and 3′ (5.6 kb downstream of the 3′ UTR of *egl-1*) regulatory regions of the *egl-1* gene (P*egl-1*::*gfp*) (Figure 1). This reporter construct was introduced into animals carrying the *ced-3*(lf) mutation *n717*. *ced-3* acts downstream of *egl-1* genetically, and *ced-3*(lf) mutations block most if not all programmed cell deaths that occur during development, including the deaths of the HSNs in males. This experimental design allowed us to analyze the expression of the reporter construct in the HSNs in both sexes. While GFP was present in a number of cells, we failed to detect the protein in the HSNs in hermaphrodites carrying extrachromosomal arrays of the reporter construct. None of the HSNs (n = 60) in transgenic hermaphrodites were positive for GFP. By contrast, we detected GFP in 87% of the HSNs (n = 60) in transgenic males (Figure 3). The *egl-1* gene therefore appears to be expressed in the HSNs in males, in which the HSNs normally undergo programmed cell death, but not in hermaphrodites, in which the HSNs normally survive.

To determine the effect of the *egl-1*(gf) mutations on the expression of *egl-1* in the HSNs, we introduced the G-to-A transition found in animals carrying the *egl-1*(gf)

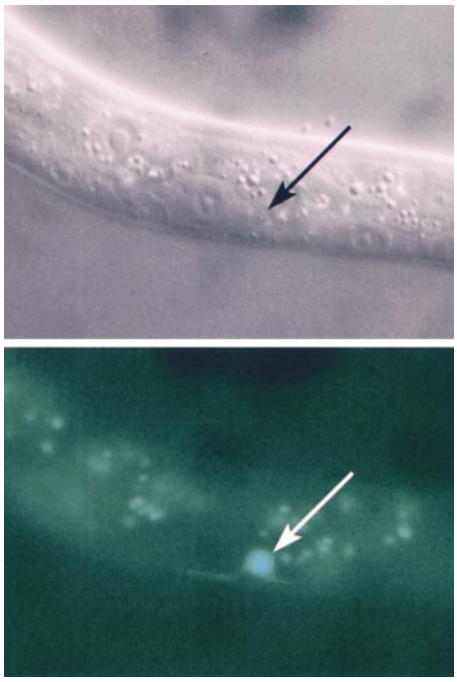


Figure 3. The *egl-1* Gene is Transcriptionally Active in the HSNs in Males

A P*egl-1*::*gfp*-reporter construct was expressed in the HSNs in males. Nomarski optics (top) and epifluorescence (bottom) images of the central region around the gonadal primordium of a *ced-3*(*n717*) male animal at the first larval stage of development carrying an extrachromosomal array of the P*egl-1*::*gfp*-reporter construct. The arrows point to the cell body of a GFP-positive HSN.

mutation *n986*, *n987*, or *n2164* (Table 1) into the TRA-1A-binding site of the reporter construct (P*egl-1*(gf)::*gfp*). This construct was introduced into *ced-3*(lf) animals. The base change resulted in the expression of *egl-1* in 78% of the HSNs (n = 120) in transgenic hermaphrodites and in 80% of the HSNs (n = 40) in transgenic males. This result demonstrates that this G-to-A transition causes the inappropriate transcriptional activation of the *egl-1* gene in the HSNs in hermaphrodites.

TRA-1A Binds to the Putative TRA-1A-Binding Site In Vitro

To determine whether TRA-1A can bind to the conserved putative TRA-1A-binding site downstream of the *egl-1* transcription unit, we performed gel mobility shift assays. We found that in vitro translated, full-length TRA-1A protein could bind to and shift a probe consisting of a 297 bp DNA fragment that we amplified from sequences 5526 to 5823 bp downstream of the *egl-1* stop codon. TRA-1A was able to bind to the probe containing the putative TRA-1A-binding site amplified from wild-type animals but failed to bind to the corresponding probes amplified from animals carrying the *egl-1*(gf) mutation *n1084*, *n2164*, or *n2248*, which represent the three different G-to-A transitions (Figure 4A).

To examine the binding of TRA-1A to the mutant TRA-1A-binding sites in more detail, we performed competition experiments. In these experiments, we used in vitro translated full-length TRA-1A protein, labeled wild-type DNA fragments as a probe, and unlabeled wild-type or mutant DNA fragments as competitors. A 10-fold excess of the wild-type competitor was sufficient to reduce TRA-1A binding to the wild-type probe by 50% (Figures 4B and 4C). TRA-1A binding to the wild-type probe was almost completely abolished in the presence of a 100-fold excess of wild-type competitor (Figures 4B and 4C). These observations are in agreement with data obtained by others using a truncated TRA-1A protein (Zarkower and Hodgkin, 1993). Competitor carrying the *egl-1*(gf) mutation *n1084* reduced TRA-1A binding to the wild-type probe by 50% when present at a 500-fold excess (Figures 4B and 4C). Fragments carrying the mutations *n2164* or *n2248* were less effective competitors than the *n1084*-containing fragment. At a 500-fold excess, the *n2164*- and *n2248*-containing fragments reduced TRA-1A binding to the wild-type probe only by about 25% (Figures 4B and 4C). This result shows that TRA-1A binds to the TRA-1A-binding sites carrying the three different G-to-A transitions, although with reduced affinities, and that TRA-1A has a higher affinity for the *n1084*-containing binding site than for the *n2164*- or *n2248*-containing binding sites.

The Different *egl-1*(gf) Mutations Have Different Effects on HSN Survival In Vivo

To determine whether the three different G-to-A transitions also behave differently in vivo, we determined their effects on HSN survival in hermaphrodites. Compared to 100% HSN survival in wild-type hermaphrodites, 0% of the HSNs survived in hermaphrodites homozygous for any of the three *egl-1*(gf) mutations *egl-1*(*n1084*gf), *egl-1*(*n2164*gf), and *egl-1*(*n2248*gf) (Table 2). However, 34% of the HSNs survived in hermaphrodites heterozygous for the mutation *n1084* (*egl-1*(*n1084*gf)/+), and 11% and 9% of the HSNs survived in hermaphrodites heterozygous for the mutations *n2164* and *n2248*, respectively (*egl-1*(*n2164*gf)/+, *egl-1*(*n2248*gf)/+). The survival rates of the HSNs in animals heterozyous for *n1084*, *n2164*, and *n2248* therefore correlate with the affinities with which TRA-1A binds to the mutant TRA-1A-binding sites in vitro. These results are consistent with the hypothesis that TRA-1A acts as a transcriptional repressor of the *egl-1* gene in the HSNs.

tra-1 Specifies the Cell Death Fate of the HSNs and Acts as a Negative Regulator of *egl-1*

To determine whether the *tra-1* gene plays a role in specifying the cell death fate of the HSNs, we analyzed the survival of the HSNs in animals carrying either lf or gf mutations in the *tra-1* gene. *tra-1*(lf) mutations lead to the masculinization of the soma of hermaphrodites (XX) (Hodgkin, 1987). Compared to 100% survival in wild-type XX animals, only 11% of the HSNs survived in XX animals homozygous for the strong *tra-1*(lf) mutation *e1099* (Table 3). Maternal effects have not been reported for the *tra-1* gene. However, that 11% of the HSNs survived in essentially male *tra-1*(*e1099*lf) XX animals might be a consequence of the presence of maternally derived active TRA-1A protein during embryogenesis, when the HSNs normally die.

Dominant gf mutations in *tra-1* cause the synthesis of a TRA-1A protein that is active in both hermaphrodites

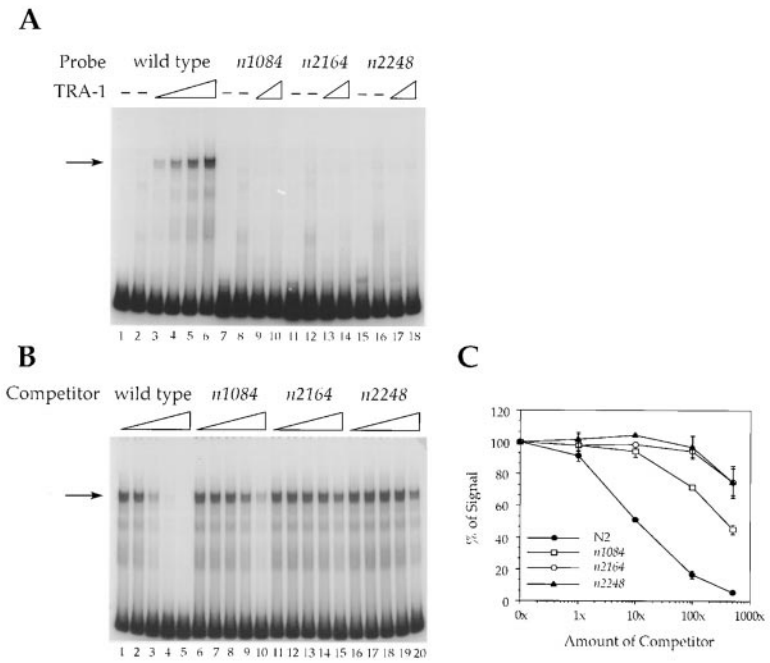


Figure 4. TRA-1A Binds to the Putative TRA-1A-Binding Site Downstream of the *egl-1* Transcription Unit

(A) TRA-1A binds to the wild-type but not the mutant putative TRA-1A-binding sites. The arrow points to the specific complexes formed by full-length TRA-1A protein and the DNA fragment used as probe. TRA-1-containing lysate (0 μl [lanes 1, 7, 11, and 15], 0.15 μl [lanes 3, 9, 13, and 17], 0.38 μl [lane 4], 0.75 μl [lane 5], or 1.5 μl [lanes 6, 10, 14, and 18]) or control lysate (1.5 μl [lanes 2, 8, 12, and 16]) was incubated with 4 ng of labeled wild-type (lanes 1–6) or *n1084*- (lanes 7–10), *n2164*- (lanes 11–14), or *n2248*- (lanes 15–18) containing DNA fragments and analyzed for gel mobility shifts as described in Experimental Procedures.

(B) DNA fragments containing the *egl-1*(gf) mutations functioned as weak competitors. The arrow points to the specific complexes formed by full-length TRA-1A protein and the DNA fragment used as probe. TRA-1A-containing reticulocyte lysate (1.5 μl) was incubated with 2 ng of labeled wild-type DNA fragments and with 0 ng (0×; lanes 1, 6, 11, and 16), 2 ng (1×; lanes 2, 7, 12, and 17), 20 ng (10×; lanes 3, 8, 13, and 18), 200 ng (100×; lanes 4, 9, 14, and 19), or 1 μg (500×; lanes 5, 10, 15, and 20) of unlabeled competitor DNA, amplified from wild-type (lanes 1–5), *egl-1*(*n1084*gf) (lanes 6–10), *egl-1*(*n2164*gf) (lanes 11–15), or from *egl-1*(*n2248*gf) (lanes 16–20) animals. Results were analyzed as described in Experimental Procedures.

(C) Comparison of the efficiencies of the various competitors. The data shown were generated as described in (B) and analyzed using a PhosphoImager. The amount of TRA-1A-DNA complex formed in the absence of competitor ([B], 0×, lanes 1, 6, 11, and 16) was used as 100% of signal for each particular data set (N2, *n1084*, *n2164*, *n2248*). N2, wild type. Data represent average ± standard deviation of two or three independent experiments.

and males, resulting in the feminization of the soma of males (X0) (Hodgkin, 1987). While 0% of the HSNs survived in wild-type X0 animals, 82% of the HSNs survived in X0 animals heterozygous for the *tra-1*(gf) mutation *e1575* (*tra-1*(*e1575*gf)/+) (Table 3). (That 18% of the HSNs in *tra-1*(*e1575*gf) animals died presumably reflects the incomplete feminization of such animals (Hodgkin, 1987), which might have been amplified by the fact that these animals were also heterozygous for a *tra-2*(lf) mutation.) Thus, both lf and gf alleles of *tra-1* affected the cell death fate of the HSNs.

To test whether the ability of *tra-1* to specify the cell death fate of the HSNs is dependent on *egl-1*, we analyzed animals carrying mutations in both genes. Ninety-six percent of the HSNs survived in XX animals that carry the *egl-1*(lf) mutation *n3082*, but only 11% of the HSNs survived in *tra-1*(*e1099*lf) XX animals. In XX animals carrying both mutations, *tra-1*(*e1099*lf); *egl-1*(*n1084 n3082*lf), 88% of the HSNs were present, suggesting that the *egl-1*(lf) mutation can suppress the lf mutation in *tra-1* (Table 3). Furthermore, only 1% of the HSNs

Table 2. The Three *egl-1*(gf) Mutations Have Different Effects on HSN Survival in Hermaphrodites

Genotype	Percent of HSNs Present (n)
+/+	100 (50)
egl-1(*n1084*gf)	0 (50)
egl-1(*n1084*gf)/+	34 (82)
egl-1(*n2164*gf)	0 (40)
egl-1(*n2164*gf)/+	11 (80)
egl-1(*n2248*gf)	0 (42)
egl-1(*n2248*gf)/+	9 (88)

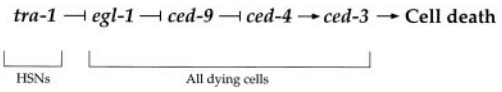
The presence of HSNs was scored by Nomarski optics (Sulston and Horvitz, 1977). The complete genotypes of the animals scored were as follows: +/*unc-76*(*e911*), *egl-1*(*n1084*) +/*egl-1*(*n1084*) *unc-76*(*e911*), *egl-1*(*n1084*)+/+*unc-76*(*e911*), *egl-1*(*n2164*)+/*egl-1*(*n2164*) *unc-76*(*e911*), *egl-1*(*n2164*)+/+*unc-76*(*e911*), *egl-1*(*n2248*)+/*egl-1*(*n2248*) *unc-76*(*e911*), *egl-1*(*n2248*)+/+*unc-76*(*e911*). n, number of HSNs.

Table 3. *egl-1* Mutations Suppress the Effects of *tra-1* Mutations on the Fate of the HSNs

	Percent of HSNs Present (n)	
Genotype	XX	XO
+/+	100 (50)	0 (46)
egl-1(*n1084 n3082*lf)	96 (52)	82 (62)
egl-1(*n2164*gf)/+	11 (80)	1 (84)
tra-1(*e1099*lf)	11 (46)	ND
tra-1(*e1099*lf); *egl-1*(*n1084 n3082*lf)	88 (46)	ND
tra-1(*e1575*gf)/+	ND	82 (52)
tra-1(*e1575*gf)/+; *egl-1*(*n2164*gf)/+	ND	8 (38)

The presence of HSNs was scored as described by Sulston and Horvitz (1977). The complete genotypes of the animals scored were as follows: +/*unc-76*(*e911*), *egl-1*(*n1084 n3082*)+/*egl-1*(*n1084 n3082*) *unc-76*(*e911*), *egl-1*(*n2164*)+/+*unc-76*(*e911*), *tra-1*(*e1099*), *tra-1*(*e1099*); *egl-1*(*n1084 n3082*), *tra-2*(*e1095*)/+; *tra-1*(*e1735*)/+; *him-5*(*e1490*)/+; +/*axIs36*, *tra-2*(*e1095*)/+; *tra-1*(*e1735*)/+; *him-5*(*e1490*)/*egl-1*(*n2164*); +/*axIs36*. ND, not determined.

A



B

Genotype	Sex	TRA-1A Activity	*egl-1*	HSN Survival
wild type	XX	HIGH	OFF	+
wild type	X0	LOW	ON	-
egl-1(*n1084*gf)	XX	HIGH	ON	-
egl-1(*n1084 n3082*lf)	XX	HIGH	ON	+

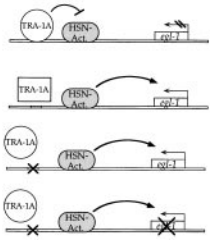


Figure 5. *tra-1* Is a Negative Regulator of *egl-1* in the HSNs

(A) The genetic pathway for programmed cell death in *C. elegans*. In the HSNs, *tra-1* acts upstream of or in parallel to *egl-1*, *ced-9*, *ced-4*, and *ced-3*, the components of the central programmed cell death pathway, to block programmed cell death.

(B) Model for the role of TRA-1A in the transcriptional regulation of *egl-1* in the HSNs. By negatively regulating an HSN-specific activator of *egl-1* transcription, TRA-1A acts as a repressor of *egl-1* in the HSNs in hermaphrodites. The absence of *egl-1* activity results in the survival of these neurons in hermaphrodites. See Discussion for details.

were present in X0 animals heterozygous for the *egl-1*(gf) mutation *n2164*, whereas 82% survived in X0 animals heterozygous for the *tra-1*(gf) mutation *e1575*. We found that only 8% of the HSNs were present in X0 animals heterozygous for both *tra-1*(*e1575*gf) and *egl-1*(*n2164*gf), *tra-1*(*e1575*gf)/+; *egl-1*(*n2164*gf)/+, suggesting that the *egl-1*(gf) mutation is able to suppress the *tra-1*(gf) mutation. These results indicate that with respect to the HSN cell death fate *egl-1* is epistatic to *tra-1* and that *egl-1* therefore acts downstream of or in parallel to *tra-1* in the HSNs. *tra-1* therefore acts as a negative regulator of *egl-1* in these neurons (Figure 5A).

Discussion

TRA-1A Represses *egl-1* Transcription in the HSNs

Based on the following observations we propose that the *egl-1* gene is regulated at the transcriptional level in the sexually dimorphic HSNs and that TRA-1A acts as a repressor of *egl-1* transcription in these neurons. (1) *egl-1*(gf) mutations cause the deaths of the HSNs in hermaphrodites by inappropriately activating the pathway of programmed cell death (Trent et al., 1983; Ellis and Horvitz, 1986). (2) These gf mutations affect a conserved TRA-1A-binding site near the *egl-1* transcription unit, and this site is required in *cis* to the *egl-1* transcription unit for the proper regulation of the *egl-1* gene in the HSNs. (3) The *egl-1*(gf) mutations strongly reduce the ability of TRA-1A to bind to this site in vitro and cause the inappropriate transcriptional activation of the *egl-1* gene in vivo. (4) The *tra-1* gene acts genetically as a negative regulator of *egl-1* in the HSNs.

Figure 5B illustrates how the transcription of the *egl-1* gene might be regulated in the sexually dimorphic HSNs. In wild-type hermaphrodites (XX), TRA-1A activity is high, and the binding of TRA-1A to the TRA-1A-binding site downstream of the *egl-1* transcription unit negatively regulates an HSN-specific activator of *egl-1* transcription (see below), resulting in the repression of the locus and HSN survival. In wild-type males (X0), the level of TRA-1A activity is too low to negatively regulate the HSN-specific activator, which allows the activation of the *egl-1* gene and the deaths of the HSN. In *egl-1*(gf) hermaphrodites, TRA-1A activity is high but TRA-1A is unable to negatively regulate the HSN-specific activator because its binding site is disrupted, resulting in the inappropriate activation of *egl-1* in the HSNs in hermaphrodites and their deaths in the sex in which they normally survive. In *egl-1*(*n1084 n3082*lf) hermaphrodites (*egl-1*(lf)), the *egl-1* gene is also inappropriately activated, but because the *egl-1* coding region carries a mutation, the lf mutation *n3082*, an inactive gene product is made which is unable to induce programmed cell death.

The inability of TRA-1A to repress *egl-1*, as occurs in wild-type males as a consequence of low TRA-1A activity or in *egl-1*(gf) hermaphrodites as a consequence of a disrupted TRA-1A-binding site, causes the HSNs but not other cells in the animal to undergo programmed cell death. Furthermore, active TRA-1A protein blocks only the deaths of the HSNs in hermaphrodites but not the deaths of other cells destined to die. We therefore propose that the regulation of *egl-1* in the HSNs involves another factor, such as an HSN-specific activator of *egl-1* expression, and that TRA-1 specifically blocks this activator.

The *egl-1* gene represents the first identified direct target of TRA-1A in the soma, where TRA-1A functions as the terminal regulator of sexual phenotype. It has been proposed that TRA-1A implements sexual fate by promoting female-specific and by suppressing male-specific programs required for sexual differentiation (Zarkower and Hodgkin, 1993). The deaths of the HSNs can be regarded as part of the male-specific program, and by repressing *egl-1* transcription in the HSNs in hermaphrodites, TRA-1A suppresses this program. The programmed cell death pathway is therefore used in the sexually dimorphic HSNs to execute sexual differentiation.

TRA-1A Represses *egl-1* at a Distance

The TRA-1A-binding site is located 5.6 kb downstream of the *egl-1* transcription unit, beyond another transcription unit, F23B12.1 (Figure 2A). F23B12.1 is encoded on the opposite strand to *egl-1*, and its gene product is predicted to be similar to serine/threonine protein phosphatases (*C. elegans* Sequencing Consortium, 1998). It is possible that the TRA-1A-binding site also affects the transcriptional regulation of F23B12.1. However, the F23B12.1 transcription unit does not appear to be present at the corresponding location with respect to the *egl-1* locus of *C. briggsae* (our unpublished observations), supporting the hypothesis that the TRA-1A-binding site acts to regulate *egl-1*.

DNA-binding sites that act at a distance have also been characterized for the *Drosophila* Ci protein and the vertebrate Gli protein, two other members of the TRA-1A/Ci/Gli family of transcription factors. Ci acts as a transcriptional activator of the *patched* and *wingless* genes in vivo through three Ci-binding sites about 600 to 750 bp upstream of the transcriptional start site in the case of the *patched* gene (Alexandre et al., 1996) and through four Ci-binding sites 3.7 to 4.7 kb upstream of the transcriptional start site in the case of the *wingless* gene (von Ohlen et al., 1997). A single binding site for the mammalian Gli protein located 6 kb downstream of the transcription unit encoding hepatic nuclear factor-3β (HNF-3β) has been found to be required for correct HNF-3β expression (Sasaki et al., 1997).

Ci and Gli proteins have been shown to act as repressors and activators of transcription in vivo (reviewed by Johnson and Scott, 1998). It remains to be determined whether TRA-1A can act not only as a repressor but also as an activator of transcription in *C. elegans*.

The Dominant *egl-1*(gf) Phenotype Is a Result of the Loss of Binding of a Repressor

The *egl-1*(gf) mutations, which are single-base changes in a TRA-1A-binding site, strongly reduce the ability of TRA-1A to bind to this site in vitro. The loss or reduction of TRA-1A binding results in the inappropriate activation of *egl-1*, which accounts for the dominant nature of the *egl-1*(gf) mutations. There are only a few examples in multicellular organisms in which the loss of binding of a transcriptional repressor results in a dominant phenotype. The *abd-A* (*abd*-*A*, *abd*ominal *A*) gene of the *Drosophila bithorax* complex is required for the specification of the identity of the second through fourth abdominal segments (A2–A4). Mutations that disrupt the binding site for the transcriptional repressor Krüppel in an intron of the *abd-A* transcription unit (Hab mutations, hyperabdominal) cause ectopic expression of *abd-A* in the third thoracic segment (T3), which results in the dominant transformation of T3 toward A2 (Shimell et al., 1994). Similarly, dominant gf mutations in the promoter 1 (P1) of the *C. elegans* gene *her-1* (*her*, *her*maphroditization), an upstream gene in the regulatory pathway for sex determination, are thought to disrupt a binding site for the gene product of the *sdc-1* gene (*sdc*, *s*ex and *d*osage *c*ompensation), a protein containing seven zinc fingers (Nonet and Meyer, 1991) and that acts as a negative regulator of *her-1* (Trent et al., 1991; Perry et al., 1994).

Transcriptional Regulation of *egl-1* Might Integrate Cell Death Regulatory Signals

It is possible that the activity of *egl-1* is regulated at a transcriptional level not only in the HSNs but also in all other somatic cells. Our preliminary data suggest that the *egl-1* gene is transcriptionally active specifically in cells that are destined to die during development (our unpublished observations). The transcriptional regulation of *egl-1* might therefore be complex. Consistent with this hypothesis is our observation that extensive regions of identity, suggestive of extensive *cis* regulatory regions, exist between the *C. elegans* and the *C. briggsae* sequences downstream of the *egl-1* transcription unit (our unpublished observations).

Regulation at the transcriptional level might also be the mechanism by which the activity of the mammalian protein DP5 (Imaizumi et al., 1997), another member of the subfamily of BH3 domain–only cell death activators, is controlled. The transcriptional upregulation of the DP5 gene in cultured sympathetic neurons after the removal of nerve growth factor coincides with the programmed deaths of these cells (Imaizumi et al., 1997). Other mammalian BH3 domain–only cell death activators, Bad and Bid for example, appear to be regulated at the posttranslational level. The ability of Bad to bind to and thereby negatively regulate Bcl2-like cell death inhibitors depends on its state of phosphorylation, which is regulated by the presence or absence of extracellular survival factors via the phosphoinositide 3-kinase/Akt signal transduction cascade (reviewed by Franke and Cantley, 1997). Bid appears to be activated through specific Caspase 8–dependent cleavage following cell death signaling through the tumor necrosis factor-receptor or through Fas (Li et al., 1998; Luo et al., 1998).

egl-1 Acts at an Interface between Pathways that Specify Cell Fate and that Execute a Specific Cell Fate

We have shown that the *egl-1* gene is under the direct control of the sex determination pathway. *egl-1* may also be the target of the *ces-1* and *ces-2* (*ces*, *c*ell death *s*pecification) pathway, which is involved in the cell death specification of the sister cells of the NSM neurosecretory motor neurons (Ellis and Horvitz, 1991; Metzstein et al., 1996) and which appears to act upstream of *egl-1* genetically (Conradt and Horvitz, 1998). Thus, *egl-1* may generally act at the interface between pathways that specify cell fate during *C. elegans* development and a pathway that is involved in the execution of a cell fate, programmed cell death. Such a role is similar to that of the *Drosophila* gene *string*, which encodes a CDC25-type tyrosine protein phosphatase required for cell cycle progression (reviewed by Follette and O'Farrell, 1997). The pattern of embryonic cell division cycles during *Drosophila* embryogenesis is determined by pattern-forming genes that transmit developmental cues, such as positional information, to the *string* locus. This pattern is then executed through the transcriptional activation of the *string* gene, the product of which triggers mitosis (Edgar et al., 1994). Like the *string* locus of *Drosophila*, the *egl-1* locus of *C. elegans* might integrate various developmental signals and translate these signals into action, that is, the execution of another cell division cycle in the case of *string* and the execution of programmed cell death in the case of *egl-1*.

An analysis of the regulation of the *egl-1* gene might identify the pathways that determine the fate of programmed cell death in all somatic cells that are destined to die during *C. elegans* development. Such studies should help reveal how multiple developmental signals are integrated and how the process of programmed cell death is used during development not only to create sexual dimorphism within the nervous system but more generally to form a functional organism.

Experimental Procedures

General Methods and Strains

C. elegans strains were cultivated at 20°C unless otherwise noted as described by Brenner (1974). The strain N2 (Bristol) was used as wild-type strain. Mutations used in this study are listed below and are described by Riddle et al. (1997), except where noted otherwise. LGI: *ced-1*(*e1735*). LGII: *tra-2*(*e1095*). LGIII: *tra-1*(*e1575*), *tra-1*(*e1099*), *unc-69*(*e587*). LGIV: *ced-3*(*n717*). LGV: *rol-4*(*sc8*), *him-5*(*e1490*), *egl-1*(*n487*), *egl-1*(*n986*), *egl-1*(*n987*), *egl-1*(*n1084*), *egl-1*(*n1796*), *egl-1*(*n2164*), *egl-1*(*n2248*) (J. Yuan et al., unpublished data), *egl-1*(*n1084 n3082*) (Conradt and Horvitz, 1998), *unc-76*(*e911*). LGX: *lin-15*(*n765*); *axIs36* (P*pes-10*::*gfp*; M. Wallenfang et al., personal communication).

Four-Factor Mapping

Standard genetic techniques were used to separate the *egl-1* mutations *n1084* and *n3082* on LGV (Brenner, 1974). Briefly, recombinants between *rol-4* and *unc-76* were obtained by screening progeny of *rol-4*(*sc8*) ++ *unc-76*(*e911*)/+ *egl-1*(*n1084 n3082*) + or *rol-4*(*sc8*) *egl-1*(*n1084 n3082*) *unc-76*(*e911*)/++++ animals for Rol non-Unc and Unc non-Rol animals. Homozygous recombinants were analyzed for the presence of *n1084* by scoring adult hermaphrodites for egg-laying defects (Egl phenotype) and for the presence of *n3082* by analyzing the anterior pharynx of larvae at the fourth larval stage for defects in programmed cell death (Ced phenotype), as described previously (Conradt and Horvitz, 1998).

Transgenic Animals

Germline transformation was performed as described by Mello and Fire (1995). For transformation with DNA fragments amplified by PCR, *ced-1*(*e1735*); *egl-1*(*n1084 n3082*) *unc-76*(*e911*) animals were injected with gel-purified PCR fragments (2 ng/μl), and the coinjection marker p76-16B (50 ng/μl), which rescues the *unc-76*(*e911*) uncoordinated phenotype (Bloom and Horvitz, 1997). Non-Unc transgenic F1 animals were picked and used to establish lines. The rescue of the Ced phenotype and the presence of the HSNs were determined in transgenic animals at the first larval stage as described (Conradt and Horvitz, 1998). The DNA fragments were amplified by PCR using a polymerase mix suitable for long-range PCR (Clontech) and appropriate primers. Chimeric fragments were generated by digesting fragments with PstI and subsequent ligation of appropriate fragments. For transformation with GFP reporter constructs, *ced-3*(*n717*); *lin-15*(*n765*) animals were injected with plasmids pBC99 (P*egl-1*::*gfp*) or pBC104 (P*egl-1*(gf)::*gfp*) (2 ng/μl) and the coinjection marker pL15-EK (50 ng/μl), which rescues the *lin-15*(*n765*) multivulva phenotype (Clark et al., 1994). Injected animals were shifted to 24°C, and non-Muv transgenic F1 animals were picked to establish lines, which were maintained at 24°C. To construct pBC99, bases +174 to +5820 (5′-3′) downstream of the stop codon of the *egl-1* gene and bases −1914 to −837 (5′-3′) upstream of the stop codon were amplified with appropriate primers and cloned into the SpeI-ApaI (5′-3′) and PstI-BamHI (5′-3′) sites of vector pPD95.69, respectively (A. Fire et al., personal communication). pBC104 was generated from pPC99 by introducing a G-to-A transition at bp +5635 downstream of the stop codon of the *egl-1* gene using PCR-mediated mutagenesis and appropriate primers. The nucleotide sequences of all cloned fragments that were amplified by PCR were determined.

Molecular Analysis

Standard molecular biological procedures were employed (Sambrook et al., 1989), unless otherwise noted. Primers used throughout this study were based on the sequence of the *egl-1* locus as determined by the *C. elegans* Sequencing Consortium (1998). The sequences of the *egl-1*(gf) mutant alleles were determined from PCR products amplified from genomic DNA isolated from the various gf mutants. DNA sequences were determined using an automated ABI 373 DNA sequencer (Applied Biosystems). The *C. briggsae egl-1* locus was identified by probing a *C. briggsae* fosmid grid (Genome Systems) with the *egl-1* cDNA. The sequence of the positive clone (fosmid G27D19) was determined by the *C. elegans* Sequencing Consortium.

Analysis of *gfp* Expression in HSNs

The HSNs were identified in transgenic larvae at the first larval stage using Nomarski optics as described (Sulston and Horvitz, 1977) and analyzed for *gfp* expression using epifluorescence. The sex of the larvae was determined by the positions of the coelomocytes relative to the gonadal primordium and by the appearance of the nuclei of the B and C ectodermal cells in the tail (Sulston and Horvitz, 1977). Data presented in the text were from one representative line each (P*egl-1*::*gfp* and P*egl-1*(gf)::*gfp*). Similar results were obtained with three (P*egl-1*::*gfp*) and four (P*egl-1*(gf)::*gfp*) additional independent lines, respectively.

Gel Mobility Shift and Competition Experiments

For gel mobility shift experiments, probes were generated by PCR amplification at low dATP concentration (20 μM) and in the presence of 20 μCi [^{32}P] dATP, using primers #15 (5′-CTCTGTTCCAGCTCAAA TTTC-3′) and #46 (5′-GTCGTAACAAGTATCAGGCG-3′). Labeled PCR products were purified on a 6% acrylamide/TBE gel. TRA-1A protein was generated by in vitro transcription and translation of a full-length *tra-1* cDNA (pDZ118; D. Zarkower, personal communication) and a T7-based coupled reticulocyte lysate (TNT Coupled Reticulocyte Lysate System, Promega). Gel mobility shift assays were performed as described (Pollock and Treisman, 1990; Zarkower and Hodgkin, 1993) and were incubated for 20 min at room temperature before electrophoresis on 4% acrylamide gels/0.5× TBE. Unless otherwise noted, the assays contained 1.5 μl of reticulocyte lysate (source of TRA-1A) and 4 ng of labeled PCR fragments (probe). Competitor DNA was generated by PCR amplification using primers #15 and #46 and appropriate templates. PCR products were gel purified. Competition experiments were performed with 2 ng of labeled PCR fragments as probe and 0, 2, 20, 200, or 1000 ng of unlabeled PCR fragments as competitor.

Analysis of HSN Survival in *tra-1* Mutants

tra-1(*e1099*lf) XX animals were obtained and analyzed as follows. Non-Unc L1 progeny of hermaphrodites of genotype *tra-1*(*e1099*lf)/*unc-69*(*e567*) were picked and analyzed for the presence of HSNs as described (Conradt and Horvitz, 1998) and recovered and allowed to mature. Homozygous *tra-1*(*e1099*lf) XX animals were recognized by their male somatic phenotype. *tra-1*(*e1099*lf); *egl-1*(*n1084 n3082*lf) XX animals were obtained from *tra-1*(*e1099*lf)/*unc-69*(*e567*); *egl-1*(*n1084 n3082*lf) mothers. *tra-1*(*e1575*gf)/+ X0 animals were obtained by mating fertile spermless females of genotype *tra-2*(*e1095*); *tra-1*(*e1575*gf)/+; *him-5*(*e1490*) with males of genotype *axIs36*/0 (*axIs36* is the construct P*pes-10*::*gfp* integrated on the X chromosome. Paternally inherited, *axIs36*-derived GFP is therefore a marker for XX cross-progeny; M. Wallenfang et al., personal communication). L1 progeny were analyzed for the presence of HSNs and *axIs36* (GFP), recovered, and allowed to mature. *tra-1*(*e1575*gf)/+ X0 animals were recognized among GFP$^-$ animals by their female somatic phenotype. To obtain *tra-1*(*e1575*gf)/+; +/*egl-1*(*n2164*gf) X0 animals, we crossed *tra-2*(*e1095*); *tra-1*(*e1575*gf)/+; *him-5*(*e1490*) hermaphrodites with *egl-1*(*n2164*gf); *axIs36*/0 males.

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